Marker Development for Marker Assisted Breeding of Resistance to *Phytophthora Sojae* in Soybean

Rhiannon Schneider

Honors Thesis

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Introduction

One of the most destructive diseases of soybeans (*Glycine ma*x) is Phytophthora root and stem rot, which can cause enormous yield loss to an economically important field crop. Annual yield losses of nearly $200 million dollars in soybean have been attributed to this disease (Sandhu *et al*., 2005). Phythophthora root and stem rot is caused by the pathogen, *Phytophthora sojae*, an oomycete that is closely related to Brown Algae but shares similar characteristics with Fungi. This pathogen thrives in wet climate conditions and poorly drained soils and can be difficult and expensive to control when using chemical treatments. The use of soybean cultivars with resistance to *P. sojae* is a more efficient approach in controlling this pathogen.

There are fourteen different resistance genes to *P. sojae,* known as *Rps* genes, which have been identified from the soybean germplasm and confer dominant race-specific resistance. *Rps1a, b, c, d,k,* and *Rps7* are mapped to chromosome 3 (Lohnes and Schmitthenner, 1997;Diers *et al., 1992*). *Rps2* is mapped to chromosome 16 (Graham, *et al*., 2002). *Rps3a, b, c* and *Rps8* are mapped to chromosome 13 (Sandhu *et al.,* 2005).*Rps4*, *Rps5*, and *Rps6* are mapped to chromosome 18(Diers *et al.,* 1992; Demirbas *et al.,* 2001). *Rps1c, Rps1k, Rps3, Rps6*, and *Rps8* are utilized in the OSU-OARDC soybean breeding program.

Each *Rps* gene or allele confers resistance to a specific race or combination of races of *P. sojae*. Due to selection pressures, the *P. sojae* races are capable of mutating into a virulent strain through alteration or loss of avirulence genes. The virulent strain is immune to a particular *Rps* gene. Stacking or combining multiple *Rps* genes together provides a more durable resistance against the virulent strains. In a breeding program, the selection of multiple *Rps* genes which confer resistance to the same races of *P.sojae* requires the use of molecular markers.

Dominant race-specific genes have been commonly found to genetically co-localize with a known class of resistance genes, nucleotide binding site- leucine rich repeat (NBS-LRR) encoding genes. NBS-LRR encoding genes belong to one of the largest protein families and over 400 are predicted in soybean (Zhang *et al.,* 2010). Studies have shown NBS-LRR proteins to play a role in the plant's defenses against pathogens through signaling pathways. (McHale *et al*.,2006). *Rps1k* has been found to co-locate with two NBS-LRR encoding genes, Rps1-k-1 and Rps1-k-2 (Gao and Battacharyya, 2008). *Rps4* has also shown to co-locate with a NBS-LRR encoding gene (Sandhu *et al.,* 2004). According to the sequenced soybean cv. 'Williams 82' genome (Schmutz *et al.,* 2010) all *Rps* genes co-localize with at least one of the NBS-LRR encoding genes. NBS-LRR encoding genes are hyper variable and are potentially useful as molecular markers .There are 61 NBS-LRR encoding genes that co-localized with the *Rps* loci used in the OSU-OARDC soybean breeding program (figure 1). In addition, there are eight

microsatellites which were previously identified as linked to the resistance genes (Demirbas *et al*., 2001).

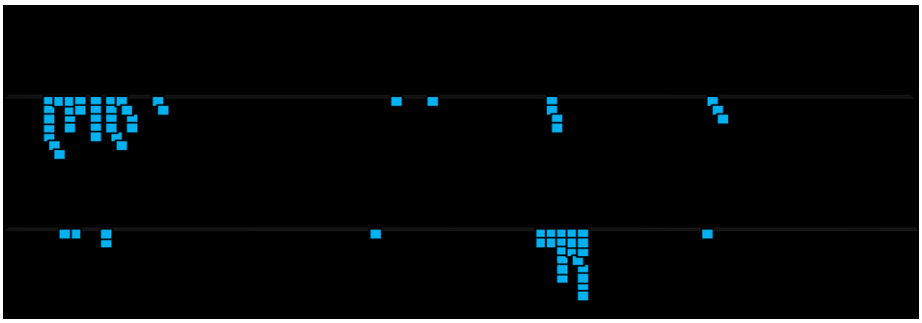

Figure 1. Soybean chromosomes containing the relevant *Rps* loci. NBS-LRR encoding genes are represented by blue squares.

The purpose of this research project is to ultimately improve efficiency in the OSU-OARDC breeding program. Current breeding methods for selecting *P. sojae* resistant soybean lines involves time consuming disease assays and requires a large amount of seed and space. Disease assays are very labor intensive and can only evaluate a small number of soybean lines at a time. Selection of resistant lines at the F_4 generation requires susceptible lines to be also maintained in the breeding program that are not useful. Phenotypic disease assays also have a limitation of only being able to effectively screen for a single *Rps* gene, but not for multiple *Rps* genes conferring resistance to the same race of the pathogen. A more efficient approach is to use molecular markers for selecting resistant lines in earlier generations. Marker assisted selection (MAS) can help improve breeding efficiency by screening a large number of F_2 individuals and selecting for only the homozygous resistant lines to advance to yield trials. In addition, molecular makers can also screen for multiple *Rps* genes that will allow the combination of these genes for more durable resistance. Although linked microsatellite markers have been developed for *Rps* genes, they are often not polymorphic within the OSU-OARDC breeding material and, therefore, not informative for making selections in a breeding program. This obviates the need for more informative, high-throughput molecular makers that will improve the efficiency of our breeding program by increasing the number of new *P.sojae* resistant soybean cultivars. Developing molecular marker would also benefit future studies for identifying and cloning *Rps* genes and alleles that have not yet been cloned.

It is hypothesized that sequence polymorphism within the genetically linked NBS-LRR encoding genes or the eight tightly linked satellite markers will be exactly correlated with the resistant phenotype. The objective was to sequence the genetically linked NBS-LRR encoding genes and the eight tightly linked satellite markers and to test for correlations to the resistant phenotype.

Table 1. Soybean cultivars from which NBS-LRR encoding genes and microsatellite markers have been sequenced.

Soybean Cultivar	*Rps* allele
Williams 82	*Rps1k, rps3, rps8*
Dennison	*Rps1k, Rps3a, rps8*
General	*Rps1k, rps3, rps8*
OHS 305	*Rps1k, Rps3a, rps8*
HS0-3243	*Rps1k, Rps3a, rps8*
Kottman	*Rps1k, Rps3a, rps8*
Streeter	*Rps1c,Rps3a,rps8*
OHS304	*Rps1c,Rps3a, rps8*
Amsoy 71	*Rps1c, rps3, rps8*
HFPR-3	*Rps1k,Rps3a,Rps8*
Wyandot	rps1, *Rps3a, rps8*
PrOhio	*rps1, rps3, rps8*
OHS 202	*rps1, rps3, rps8)*

Materials and Methods

Plant material and DNA extractions

The thirteen soybean cultivars (listed in Table 1.) were grown in the Howlett Greenhouse (The Ohio State University, Columbus, OH). Leaf tissue from the first true leaves were sampled and placed into 2.0 ml micro centrifuge tubes (USA Scientific, Ocala, FL), frozen in liquid nitrogen, and stored in -80°C freezer. The DNA extraction protocol was adapted from Kim, *et al*., 1988. Tissue was ground directly in liquid nitrogen using a mortar and pestle and pipetted (BioExpress pipette tips, Kaysville, UT) into a 2 ml tube or tissue was ground by adding ball bearings to the 2 ml tubes and placing the tubes into the TissueLyser II for 30 cycles per second for 1 minute. DNA extraction buffer was prepared on the same day of DNA extractions with a final concentration of 2% CTAB (Hexadecyl trimethyl-ammonium bromide), 100mM of Tris (pH 8.0), 20mM EDTA (EthylenndiaminetetraAcetic acid Di-sodium salt, pH 8.0), and 1.4 mM of NaCl. 700 µl of fresh extraction buffer was added to each tube and mixed by the analog vortex mixer (VWR International, Radnor, PA). The tubes were placed into a 65°C water bath (327 Kottman Hall) for 30 minute, tubes were centrifuged at 10,200 rpm for 15 minutes in the Centrifuge 5430 R (Eppendorf, Hauppauge, NY). Supernatant 500 µl was transferred to a 1.5 ml micro centrifuge tube (Fisher Scientific, Pittsburgh, PA) and 500 µl of 24:1 chloroform/isoamyl alcohol was added. The tubes were centrifuged at 10,200 rpm for 15 minutes; the aqueous phase was transferred 200 µl to new 1.5 ml tubes. DNA was precipitated in 50% isopropanol, mixed gently by inversion, and incubated at room temperature for 15-30 minutes. The tubes were centrifuged at 13,200 rpm for 15 minutes to pellet DNA. Supernatants were decanted and the pellet was washed by adding 400 µl of 70% ethanol followed by centrifugation at 10,200 rpm for 15 minutes. Supernatant was decanted and ethanol allowed to evaporate at 37°C for ~45 minutes. DNA was re-suspended in 100 µl of Tris-EDTA (TE buffer). Quality and concentration of DNA were assessed by gel electrophoresis and the Nanodrop.

Amplification and sequencing of products

Primers were designed using *Primer3* Input v.0.4.0 software (Rozen and Skaletsky, 2000) to amplify the intron regions of the genetically linked NBS-LRR encoding genes (Table 2). The primers were synthesized in two 96-well plates (Eurofins MWG Operon, Huntsville, AL).

Polymerase chain reactions were performed using the designed primers and the genomic DNA in a 96-well plate (BioExpress).Reagent used in the PCR were iProof HF Buffer with a final concentration of 1X (Bio-Rad Laboratories, Hercules,CA), dNTP with a final concentration of 200 µM each(Fischer Scientific), F primer with a final concentration of 0.5 µM(Eurofins MWG Operon), R primer with a final concentration of 0.5 µM(Eurofins MWG

Operon), 50 ng of gDNA, and iProof DNA polymerase with final concentration of 0.02 U/µl (Bio-Rad Laboratories). Thermal cycling conditions were initial denaturation of 98 °C for 30 seconds, followed by denaturation of 98 °C for 5 seconds, annealing of 60 °C for 10 seconds, and extension of 72 °C for 25 seconds, with a final extension of 72 °C for 5 minutes. Thermocyling was carried out in the MastercylcerPro (Eppendorf). The PCR products electrophoresed in 1% agarose with 0.25X concentrated GelRed and visualized by UV. PCR which failed to result in any visually observable products were repeated to eliminate chance of experimental error.

To remove excess olignoucleotides prior to sequencing, PCR product was diluted by 5 and incubated at 37 °C for 15 minutes, following 80 °C for 15 minutes with 2.5 U/ul Exonuclease I and 0.5 U/ul Shrimp Alkaline Phosphatase (SAP) (USB corporation, Cleveland, OH). The forward primer was added to 5µl of products at final concentration of 5 pmoles. Sequencing was conducted on a [name of sequencer, check PMGF website] by the Plant Microbe Genetics Facility (The Ohio State University, Columbus, OH http://pmgf.osu.edu).

Sequence analysis

Sequence analysis of 61 NBS-LRR encoding genes and the eight tightly linked microsatellite markers that co-locate or are linked to *Rps1, Rps3, Rps6, & Rps8* was done using Sequencher version 4.1 (Gene Codes corporation, Ann Arbor, MI). The sequences from the thirteen soybean cultivars were assembled into contigs for each amplicon. Sequences were grouped by resistance phenotypes. Each contig containing the thirteen sequences represented one NBS-LRR encoding gene. Polymorphisms were checked visually and counted manually.

Results

Analysis of polymorphism between resistant phenotypes has been completed for 88 amplicons from 50 of the NBS-LRR encoding genes. The remaining NBS-LRR encoding genes will be analyzed once sequencing results are finished. The sequences from the eight tightly linked microsatellite markers (Table 3) resulted in no polymorphism which differentiated the resistant phenotypes.

Fifty out of the sixty one NBS-LRR encoding genes (Table 2) have been analyzed. A total of thirty-three genes were amplified in all thirteen products. Eight genes (*Glyma03g05370, Glyma13g25420, Glyma12g25440, Glyma13g26000, Glyma13g26450, Glyma13g26000, Glyma13g26140, and Glyma13g26650*) were amplified in only a subset of the thirteen genotypes. Nine of the NBS-LRR encoding genes (*Glyma03g04080, Glyma03g04180, Glyma03g04810, Glyma03g04590, Glyma03g05260, Glyma03g05350, Glyma13g25970, Glyma13g26230, and Glyma13g26530*) had multiple gene products. In total, 466 amplicons were sequenced, sequence analysis. Sequence analysis revealed 1,122 polymorphisms in 292 kb of sequence. This equates to polymorphism rate of 0.0038 or 1 nucleotide per 263 bases.

Several *(Glyma13g25420,* and *Glyma13g26420*) single nucleotide polymorphisms (SNPs) were correlated to the genotypes for the specific *Rps* gene, however not all genotypes were amplified during PCR. The "C" nucleotide at position 111 from amplicon 1, *Glyma13g25420* is correlated with *Rps3a*. The "A" nucleotide at position 5809, a deletion at position 5844, 5846, 5847, and 5873 to5877 from amplicon 5, Glyma13g26420 is correlated with *Rps3a.* In addition, one of the genotypes with the correlated SNPs (*Glyma25420)* had evidence of multiple nucleotides present at a single position (figure 2). The remaining 1,114 SNPs did not correlate to any specific *Rps* gene.

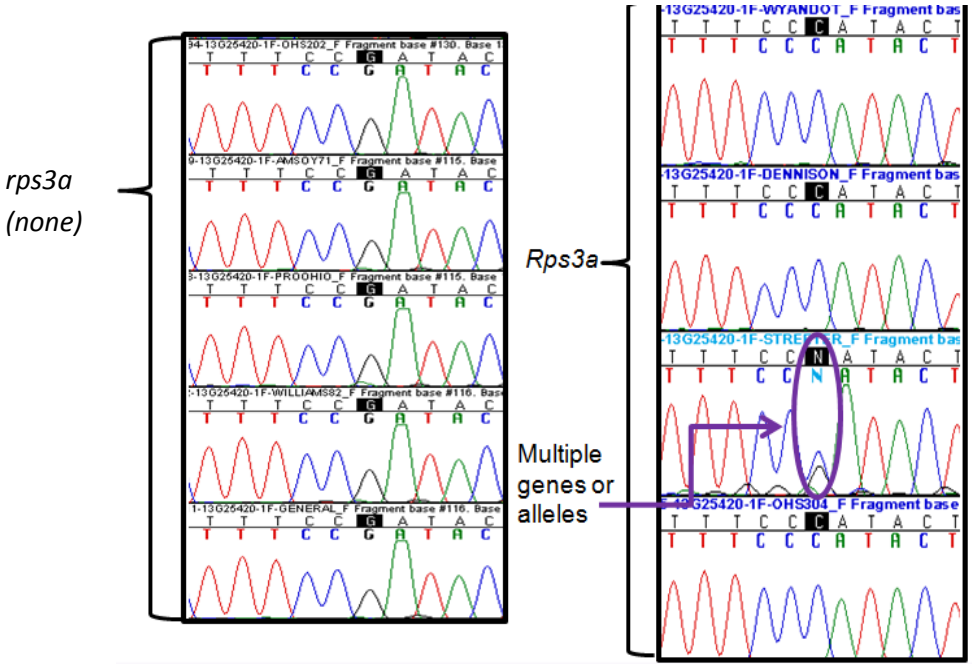

Figure 2. Chromatogram of sequences from the NBS-LRR encoding gene Glyma13g25420

Table 3. List of eight tightly linked microsatellite markers with their primer sequence (Demirbas *et al.*, 2001).

Glyma03g04030F	CTTGTTGGGAGGTGTGGAGT	1
Glyma03g04030R	AATTTGAGGGTGCTTCATGC	1
Glyma03g04080_1F	GAGATGCCGAGAGGAATGAG	1
Glyma03g04080_1R	CGGAGACAAGTTGGTTGGAT	1
Glyma03g04080_2F	TTTCCAGGTGGTCGTTTACC	1
Glyma03g04080_2R	ACCTTAATGCCAGGGATGTG	1
Glyma03g04100_1F	GTTGGAGCTGTGCTTGATGA	1
Glyma03g04100_1R	GGCAATCCATTGCACTTTTT	1
Glyma03g04100_2R	GTTTGTGTTGCTTCGGGAAT	1
Glyma03g04140R	CGACCCCAAACAGTGAGATT	1
Glyma03g04180_1R	GAGGAAAGACACGCATGGTT	1
Glyma03g04180_2F	TGGCCGAAGATCTTTTGAAG	1
Glyma03g04180_2R	TCCAACGCTTCATCACTTTG	1
Glyma03g04180_3F	TCCTACCGCAATATGACACG	1
Glyma03g04180_3R	CACATCGACCTCCAACACTG	1

Glyma03g04200_1F	GAGATGCCGAGAGGAATGAG	1
Glyma03g04200_2F	TGGGAAAAACTTGCTTCACC	1
Glyma03g04200_2R	CCCACCATATTTTCCAGCAA	1
Glyma03g04260_1F	GGAGGCAAGGATGATGGATA	1
Glyma03g04260_1R	CGCAACGATGAACTTAAGCA	1
Glyma03g04530_1F	TCAGAGTGGTTGGAGCTGTG	1
Glyma03g04530_1R	GCTGCTAAAGGCAGTCCATC	1
Glyma03g04560R	CACGCATGGTTCGTAAACAC	1
Glyma03g04610_1R	CCCCAATGTCATGCTTTCTT	1
Glyma03g04610_2R	ATACGGCATCGTTTTTCCAG	1
Glyma03g04780F	CTTGTTGGGAGGTGTGGAGT	1
Glyma03g04810R	GAGGAAAGACACGCATGGTT	1
Glyma03g05260_1F	TGTTGACTTCATCCGTGGAA	1
Glyma03g05260_1R	GCGGGTCGTCAAGAGAATTT	1
Glyma03g05260_2F	CAATAGAGGATTGCCCCAAA	1
Glyma03g05260_2R	TCTTGCAGGGATGTGAGATG	1
Glyma03g05290_1F	TGCTCAGCCAACAACATCTC	1
Glyma03g05290_1R	TGAATCAGGCAAAACATCCA	1
Glyma03g05290_2F	GGAGAATGTAACCCGAAGCA	1
Glyma03g05290_2R	TGACCCTGAACCCAAAAGAG	1
Glyma03g05350F	GTTGGAGCTGTGCTTGATGA	1
Glyma03g05350R	CAAGTGACCGTGCTGCTAAA	1
Glyma03g05370_2F	TTGTTGTGGATGGCTGAAGA	1
Glyma03g05370_2R	GGAAAAATTTCCCACCCACT	1
Glyma03g05370_3F	TAGAAGGAAGCCCAATGGTG	1
Glyma03g05370_3R	AAATTTGAGGGTGCTTCCTG	1
Glyma03g05400F	CCGCTATTGTAGGCATGGTT	1
Glyma03g05400R	CAGACTTGCAAAGCCACAAA	1
Glyma03g05420F	CTGTCTACAGATGAGGTTGTTGA	1
Glyma03g05420R	AGAAATGGTTTTGTAAGATTACTCCAA	1
Glyma03g05550F	CTCAGGTAGTTGGCCTCAGC	1
Glyma03g05550R	ACTCCACACCTCCCAACAAG	1
Glyma03g05640F	TGGGTTTGTGTTTCTGACCA	1
Glyma03g05640R	TTCATTGTTGAACCGGGAAT	1
Glyma03g05670_1F	AGGCATGAAGGGTCTTCCTT	1
Glyma03g05670_2F	TCTGATTGCGACGAGTTGAC	1

Glyma03g05670_2R	CACCATTGGGCTTCCTTCTA	1
Glyma03g05670_3F	GCTGGAGAGTATGGCTGGAG	1
Glyma03g05670_3R	CCCCTCTTATGTTTTGCATCA	1
Glyma03g05670_4F	AGCACCTTAGCGGTACATGC	1
Glyma03g05670_4R	GAGAGGATCCACTTCCCACA	1
Glyma03g05730_1F	TCTTCACGTTGGTGTTTGGA	1
Glyma03g05730_1R	CCAATAACGCGGACATCTTT	1
Glyma03g05730_2F	ATGGTCGATTATGCCAAAGG	1
Glyma03g05730_2R	CTGCATTCCATCCCAAAGTT	1
Glyma03g05730_3F	TTTGGAACTGCTTGTGCTTG	1
Glyma03g05730_3R	GGGAAGTGCAGAGAGATGGA	1
Glyma03g05880_F	GGTCGTCTCCTTTGTGGAAA	1
Glyma03g05880_R	TGGCAGCTCCTTCAAGTTTT	1
Glyma03g05890F	GATTGGTCGCATGAAGGTTT	1
Glyma03g05890R	GTGGCTTCTGAAAGGTTTGG	1
Glyma03g06210_1F	AATGATGCTGAGCTGCTTGA	1
Glyma03g06210_1R	TCAATCCATTGAAGAAACATGC	1
Glyma03g06210_2F	TTGTCGGATGCTGATGAGAC	1
Glyma03g06210_2R	GTCCAAAAGATGAGGGCAAC	1
Glyma03g06250F	GGTTTGGACCAGGCAGTAGA	1
Glyma03g06270_1F	TTCAAGCAACGTCCGTGTTA	1
Glyma03g06270_1R	CCTTTCTAATCCAACAACCACTG	1
Glyma03g06270_2F	GATTATGGGATGCCGATGAC	1
Glyma03g06270_2R	ATGCATCGAAAAGGTCCAAC	1
Glyma03g06300_1F	AGGATTGGTTGGTGTCGAAG	1
Glyma03g06300_1R	CACTTCCTGTGCAATGGTTG	1
Glyma03g06300_2F	TGCATGTTTCTGCCGTAGAG	1
Glyma03g06300_2R	TGGCAAGCTTTCAATGTCAC	1
Glyma03g06860F	TTGGAGAAACTCAAGAAAATTCC	1
Glyma03g06860R	CTGGGCTTCTTTCCACAAAA	1
Glyma03g07020F	CGGGATTGCCACTAGCTCT	1
Glyma03g07020R	TTGTTGGTATGCATGCTAAAGG	1
Glyma13g25420_1F	GACAACATATGCGTCGTTGG	3 & 8
Glyma13g25420_1R	ATTCCCATTCTTCCCATTCC	3 & 8
Glyma13g25420_2F	TAAAGGGCTTGATGGGATTG	3 & 8
Glyma13g25420_2R	AGAGTGATTGCCTCCCAATG	3 & 8

Glyma13g25440_1F	TGGGGGTAAACAATTTCCAA	3 & 8
Glyma13g25440_1R	ACGACACGCGTTTAATGTGA	3 & 8
Glyma13g25750_1F	CGCGTTCCTTATGTGATCCT	3 & 8
Glyma13g25750_1R	CAAGAAGAAGCATGCAACCA	3 & 8
Glyma13g25780_1F	CAAATGGATTCCCAGCATCT	3 & 8
Glyma13g25780_1R	AGACGCAGCATTTTGTCCTT	3 & 8
Glyma13g25780_2F	GGCCGCAGATTTGAAAAATA	3 & 8
Glyma13g25780_2R	CTCCTTCGGGAAACATTTGA	3 & 8
Glyma13g25920_1F	CCTCTGGCCTTGACAACAAT	3 & 8
Glyma13g25920_1R	AATTCAAGGCGATGCAAATC	3 & 8
Glyma13g25950_1F	ACCAAATCCAGATTGCAAGG	3 & 8
Glyma13g25950_1R	CCAGTTTTCCCAAATGTGCT	3 & 8
Glyma13g25970_1F	CAAAATGCAAGTGGTGTTGG	3 & 8
Glyma13g25970_1R	GGAATAAGGCGCAATAAGCA	3 & 8
Glyma13g25970_2F	ATGCAAGTGGTGTTGGATCA	3 & 8
Glyma13g25970_2R	AAGCAGATGTCCCCACAAAC	3 & 8
Glyma13g25970_3F*	GGAATGGGAAGAATGGGAAT	3 & 8
Glyma13g25970_3R	CCTGGGGCAGTCATAAAGAA	3 & 8
Glyma13g26000_1F	CATCCTTGGAATCTTTGGATTT	3 & 8
Glyma13g26000_1R	TCCACGTCTTCAATGTGAGC	3 & 8
Glyma13g26140_1F	AGTCGTCTGTTTCGGAATGG	3 & 8
Glyma13g26140_1R	AATGCATTGGCACCTTTCTC	3 & 8
Glyma13g26140_2R	AGTGAGAGAGGGTGGAAGCA	3 & 8
Glyma13g26230_1F	GAGCTATCCCCACCTTCCTC	3 & 8
Glyma13g26230_1R	GATTTGGGAAGACCCTCCTC	3 & 8
Glyma13g26250_1F	TCTGGCTGATGATGCAGAAC	3 & 8
Glyma13g26250_1R	ATCTCGCTCTGCCATATGCT	3 & 8
Glyma13g26310_1F	TCAGAACCTGCAGAATGTCG	3 & 8
Glyma13g26310_1R	TTTGATGGCAAACCTCCTTC	3 & 8
Glyma13g26380_1F	GGAAGCTGTGCAAACTCCTC	3 & 8
Glyma13g26380_1R	AGACTCGGGCACCTCAGTAA	3 & 8
Glyma13g26400_1F	CCTTCAATCTTTCCGTCGAG	3 & 8
Glyma13g26400_1R	TCAATGCAATCCAAAACTGC	3 & 8
Glyma13g26400_2F	GGAGTCAACGTGGTTGGAGT	3 & 8
Glyma13g26400_2R	ATCCCAGAAAATTCCCCATC	3 & 8
Glyma13g26400_3F	ACCCAAAACGAAAACACCTG	3 & 8

Glyma13g26400_3R	GCCAAGTCGTTGCCAGTAAT	3 & 8
Glyma13g26400_4F	CGGTTCTGTTCCAGGGTTTA	3 & 8
Glyma13g26400_4R	GAGGATGAGCCTTTTGTGGA	3 & 8
Glyma13g26400_5F	GCCTCAAACAAAAACGGGTA	3 & 8
Glyma13g26400_5R	AGGGAGACTGCTTGAGACCA	3 & 8
Glyma13g26400_6F	CGCCCATAGAGCAAAATCAC	3 & 8
Glyma13g26400_6R	TACCTCCCCCAGTTGAGATG	3 & 8
Glyma13g26420_1F	TGGAGAAAAGGGGAATCCAT	3 & 8
Glyma13g26420_1R	ATCTCAGCAAGCAGGGTTTG	3 & 8
Glyma13g26420_2F*	AGTTTTGGCCAATGGTGAAG	3 & 8
Glyma13g26420_2R*	TCTGGCTTAAAATCGGATGG	3 & 8
Glyma13g26420_3F	CATGATTCGGTTGGATTCCT	3 & 8
Glyma13g26420_3R	GAATCCTTGTTCCTGGCAAA	3 & 8
Glyma13g26420_4F	TTGCTAGTGATGGAGGCAAG	3 & 8
Glyma13g26420_4R	AGGTGGGGACCATGAATTTT	3 & 8
Glyma13g26420_5F	TCTTTCCCTTCGCTTTTTCA	3 & 8
Glyma13g26420_5R	AACATCTCAAAGGCCCACAC	3 & 8
Glyma13g26450_1F	CAAGGCGATTAAGGAATCCA	3 & 8
Glyma13g26450_1R	ATGACTTTGCTGCCAGAACC	3 & 8
Glyma13g26450_2F	ATGATTGGGATATGCGGAGA	3 & 8
Glyma13g26450_2R	CCCGCTTCCATCATTTAAGA	3 & 8
Glyma13g26460.1_1F	CCATACCTTCATTGGCGACT	3 & 8
Glyma13g26460.1_1R	GCCAAAACTTCCACCTCGTA	3 & 8
Glyma13g26460.1_3F	TGCACAACTGTGGAATGGTT	3 & 8
Glyma13g26460.1_3R	GCATGAGTCCAGCAAGACAG	3 & 8
Glyma13g26530.1_1F	TCTGGCTGATGATGCTGAAC	3 & 8
Glyma13g26530.1_1R	TGCAATCTGGATTTGGTTGA	3 & 8
Glyma13g26650_1F	CCATTTTTCTTCGAGGTGGA	3 & 8
Glyma13g26650_1R	GCAAGCAACATGGTCAGAGA	3 & 8
Glyma13g26650_2F	AAAGGGAAAACAACTGGGAAA	3 & 8
Glyma13g26650_2R	CGAAGGTTGGATTTCTTCCA	3 & 8
Glyma13g26650_3F	GGGGGTGTATCAGTTGTTGG	3 & 8
Glyma13g26650_3R	CGGTGCTATGGTCGCTATTT	3 & 8
Glyma13g26650_4F	TGGAACAACATGCAAAAGGA	3 & 8
Glyma13g26650_4R	CCCATAGCTCCGTAGGAACA	3 & 8

Table 3. List of eight tightly linked microsatellite markers with their primer sequence (Demirbas *et al*., 2001).

Rps	Microsatellite	Primer #	Sequence
Rps1	Satt159	Satt159-F1	GAAATGCCCAGAAAAACCTAATAAC
		Satt159-R1	TGAAGCAACAAAATAGAGGAATAGAG
Rps1	Satt152	Satt152-F1	GCGCTATTCCTATCACAACACA
		Satt152-R1	TAGGGTTGTCACTGTTTTGTTCTTA
Rps1	Satt530	Satt530-F1	CATGCATATTGACTTCATTATT
		Satt530-R1	CCAAGCGGGTGAAGAGGTTTTT
Rps1	Satt584	Satt584-F1	GCGCCCAAACCTATTAAGGTATGAACA
		Satt584-R1	GCGGGTCAGAAGATGCTACCAAACTCT
Rps2	Satt547	Satt547-F1	GCGCTATCCGATCCATATGTG
		Satt547-R1	TGATTTCGCTAGGTAAAATCA
Rps3	Satt114	Satt114-F1	GGGTTATCCTCCCCAATA
		Satt114-R1	ATATGGGATGATAAGGTGAAA
Rps4	Satt472	Satt472-F1	GCGAATACATAAAACTCAAATTCAAATCATA
		Satt472-R1	GCGTTCTATAAATTTCATTCATAGTTTCAAT
Rps6	Satt191	Satt191-F1	CGCGATCATGTCTCTG
		Satt191-R1	GGGAGTTGGTGTTTTCTTGTG

Discussion

Recent work has been conducted on NBS-LRR encoding genes for development of markers for marker assisted selection (MAS). This was used in sunflower for developing molecular markers for several resistance genes to Downy Mildew and Rust in resistance gene candidates (RGC) and NBS-LRR encoding genes (Bachlava *et al*., 2011). Further work was completed involving sunflower and the development of markers for resistance genes to Rust. One of the Rust resistance genes, *R4,* was flanked by two molecular markers that were designed within a cluster NBS-LRR encoding genes. These markers were tightly linked to the *R4* gene and the authors proposed their use for gene pyramiding of Rust resistance in sunflower breeding programs. Similar work was conducted on the finger millet crop in developing molecular markers for resistance to *Magnaportha grisea* where the conserved regions of the NBS-LRR encoding genes were used for designing markers. Five of these primers identified as useful makers (Panwar et al., 2011).

The current study identified eight SNPs putatively correlated to a specific *Rps* phenotype. Further analysis will be required before deciding which sequences can be evaluated for markers. The SNPs found in the genotype conferring to the specific Rps gene, where some of the genotypes did not amplify during PCR , will be further analyzed by designing new primers that will amplify another region of the gene. If the previously non-amplified genotypes amplify with the new primers, then this could mean that the previous non-amplified primers did not bind to these genotypes due to the presence of divergent sequence between the primers and the template DNA.

While the “C” nucleotide at position 111 from amplicon 1, *Glyma13g25420* is correlated with *Rps3a* resistance, the cultivar Streeter (*Rps1c,Rps3a*) has mixed nucleotides at this position. Further analysis would also be required for the genotype that had multiple genes in (figure 2) by determining whether Streeter sample was from a mixed seed lot, if it is heterozygous at this locus, or if multiple gene copies are being amplified. This will be done by conducting phenotypic and genotypic assays on progeny derived from single plants.

The eight tightly linked microsatellite markers showed no correlation between the resistant phenotypes. While the microsatellite markers (simple sequenced repeat markers) are linked to the Rps genes, that are multiple centimorgans away (Demirbas et al., 2001). Thus, these markers are not completely linked to the *Rps* genes. Like most species, soybean exhibits highly variable linkage disequilibrium throughout the genome, with recombination occurring in within sites less than 1 kb apart (Hyten et al., 2007). This is observed even within elite North American cultivars. Also, sequence data revealed that many PCR reactions clearly resulted in

the amplification of multiple genes in a subset of individuals and seemingly a single copy in other individuals. As soybean cultivars are inbred lines. It is unlikely that so many loci are present in the heterozygote state for each cultivar. It is more likely that the primers resulted in the amplification of multiple copies in some individuals. This provides evidence for copy number variation of NBS-LRR encoding genes between individuals. Copy number variation caused by large insertions and deletions, is part of structural variation that can account for phenotypic differences between genotypes (Stankiewicz and Lupski, 2010). Clusters of NBS-LRR genes have been previously shown to exhibit copy number variation between genotypes (McHale *et al*., unpublished). Future work to determine if candidate SNPs within the NBS-LRR encoding genes are useful as diagnostic markers will involve designing makers (Taqman or another technology doe high-throughput SNP assays) based on the sequence polymorphisms ascertained from this study. Markers will be assayed on diversity of breeding lines and multiple large populations of soybean lines that are segregating for *Rps* genes and have been previously tested for resistance. Finally, these markers will be implemented in the OSU-OARDC breeding program by assaying F_2 individuals with markers and making selections on the basis of these results.

Literature Cited

Demirbas, A., Rector,B. G., Lohnes, D. G., Fioritto, R. J., Graef, G. L. et al., 2001: **Simple sequence repeat markers linked to the soybean *Rps* genes for *Phytophthora* resistance**. Crop Sci. 41: 1220–1227.

Diers, B. W., Mansur, L., Imsande, J., and Shoemaker, R. C., 1992: **Mapping *Phytophthora* resistance loci in soybean with restriction fragment length polymorphism markers**. Crop Sci. 32: 377–383.

Gao H, and Bhattacharyya, M.K., 2008: **The soybean-*Phytophthora* resistance locus *Rps1-k* encompasses coiled coil-nucleotide binding-leucine rich repeat-like genes and repetitive sequences**. BMC Plant Biol 8:29.

Graham, M.A., Marek, L.F, and Shoemaker, R.C., 2002: **Organization, Expression and Evolution of a Disease Resistance Gene Cluster in Soybean**. Genetics,162.

Haas, J.H., and Buzzell, R.I., 1976: **New races 5 and 6 of *Phytophthora megasperma var. sojae* and differential reactions of soybean cultivars for races 1 and 6.Phytopathology** 66:1361-1362.

Hyten, D.L, Choi, I.Y., Song, Q., Shoemaker, R.C., Nelson, R.L., Costa, J.M., Specht, J.E., and Cregan, P.B.,2007: **Highly Variable Patterns of Linkage Disequilibrium in Multiple Soybean Populations.** Genetics 165: 1937-1944.

Lohnes, D. G., and Schmitthenner, A. F., 1997: **Position of the *Phytophthora* resistancegene *Rps7* on the soybean molecular map**. Crop Sci. 37: 555–556.

McHale, L., Haun, W., Xu, W., Bhaskar, P., Anderson, J., Hyten, D., Gerhardt, D., Jeddeloh, J.,

Stupar, R. M, 2012: **Structural variation hotspots in soybean genome localize to clusters of biotic stress response genes.**

McHale, L., Tan, X., Koehl, P., Michelmore, R.W, 2006: **Plant NBS-LRR proteins: adaptable guards**. Genome Biology. 7: 212.1-212.11.

Rozen, S., and Skaletsky, H.J., 2000: Primer3 on the WWW for general users and forbiologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methodsand Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp365-386.

Sandhu,D. , Schallock, K.G. , Rivera-Velez, N. , Lundeen, P., Cianzio,S., andBhattacharyya, M.K., 2005: **Soybean *Phytophthor*a Resistance Gene *Rps8* Mapsclosely to the Rps Region**. Journal of Heredity. 96:536-541.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., 2010: **Genome sequence of the palaeopolyploid soybean**. Nature 14:178-83.

Zhang, X., Feng, Y., Cheng, H., Tian, D., Yang, S., Chen, J.Q., 2011: **Relativeevolutionary rates of NBS-encoding genes revealed by soybean segmental duplication**. Mol Genet Genomics. 285:79-90.